COMMERCIAL FERTILIZER N:P:K AN ALTERNATIVE NITROGEN SOURCE FOR CULTIVATION OF *SPIRULINA PLATENSIS* IN SEWAGE

DHARAM PAL and DEVINDRA V. AMLA

Plant Biotechnology Division, National Botanical Research Institute, Lucknow-226 001, India.

ABSTRACT - The cultivation of *Spirulina* on a large scale was not possible in raw sewage, unless fortified with 1% $NaHCO_3$ and a suitable nitrogen source like Na NO_3 (0.1%) or commercial fertilizer N:P:K (0.05%) which produced yields comparable to those in Zarrouk medium. The alga seems to prefer higher pH (9-10.5) and bicarbonate ions for growth. Supplementation of commercial grade fertilizer N:P:K (0.05%) in place of Na NO_3 showed an average yield ranging from 1.8-2g dry wt/l culture which was comparable to $NaNO_3$ supplemented conditions. The algal biomass obtained from N:P:K supplementation showed level of protein content, pigments and macromolecule composition similar to nitrate grown cultures. In an open-pond system, a recharging schedule of nitrogen sources for consistent optimum yield of alga with optimum protein level is worked out.

RÉSUMÉ - La culture à grande échelle de *Spirulina* dans les eaux d'égouts n'a été possible qu'en l'enrichissant avec $NaHCO_3$ (1%) et avec une source appropriée d'azote telle que $NaNO_3$ (0,1%) ou un fertilisant commercial N:P:K (0,05%) ce qui a permis d'obtenir des rendements comparables à ceux obtenus avec le milieu de Zarrouck. Les pH élevés (9-10,5) et la présence d'ions HCO_3 semblent favorables à la croissance de cette algue. L'addition d'un fertilisant du commerce N:P:K (0,05%) à la place du $NaCO_3$ a permis d'obtenir un rendement de production moyen de 1,8 à 2 g de masse sèche par litre, comparable à celui obtenu avec des cultures enrichies par le $NaNO_3$. La biomasse algale obtenue en présence de N:P:K présente des teneurs en protéines et des compositions pigmentaires et macromoléculaires semblables à celles obtenues en présence de nitrates. En bassin de culture, un schéma d'approvisionnement en azote permettant d'obtenir des rendements conséquents et optimaux en algues avec des teneurs optimales en protéine a été mis au point. (traduit par la rédaction).

KEY WORDS : *Spirulina platensis*, Zarrouk medium, N:P:K, biomass, recharging schedule, yield.

INTRODUCTION

The large scale cultivation of algae for single cell protein has been a persistent dream of biologists and engineers dating back to the famous "Carnegie Report" (Burlew, 1953). Among the green and blue-green algae, species of *Spirulina* have received greater attention as source of human food and poultry feed since it has been traditionally consumed by the tribal people in central Africa and Mexico (Shelef & Soeder, 1980). Large scale production of *Spirulina* under controlled and natural conditions have been demonstrated in France (Clement, 1975), Japan (Clement, 1978), Taiwan

(Soong, 1980), Germany (Soeder, 1978), USA (Goldman, 1979), Mexico (Durand-Chastel, 1980), and India (Saxena *et al.*, 1983; Becker & Venkataraman, 1984). Cultivation of green algae has been a successful story but its harvesting and processing have posed several technical limitations (Oswald, 1980). However, *Spirulina* has several advantages over green algae like *Chlorella, Scenedesmus* and *Coelastrum* because of high protein content (50-65%), well balanced amino-acid composition, low nucleic acid content and inexpensive harvesting procedure (Saxena *et al.*, 1983). Early cultivation of green and blue-green algae was confined to fresh waters using inorganic nutrients and high- priced nitrogenous compounds that made the production of algal biomass very expensive. Recently domestic, industrial, swine wastes and seawater supplemented with suitable nitrogen sources and bicarbonate have been used for cultivation of *Spirulina* and a reasonable yield of algal biomass for animal feed and industrial implications has been obtained (McGarry, 1971; Kosaric *et al.*, 1979; Oron *et al.*, 1974; Chung *et al.*, 1978; Faucher *et al.*, 1979; Tel-Or *et al.*, 1980). In the present investigation attempts have been made to grow the cyanobacterium *Spirulina platensis* in domestic sewage supplemented with commercial fertilizer N:P:K instead of expensive $NaNO_3$ with the view to utilize domestic waste for algal production and to minimize the production cost as well as environmental hazards.

MATERIAL AND METHODS

Organism and culture conditions - A strain of *Spirulina platensis* (ATCC 29408) obtained from Prof. Riplay D. Fox (France) was used in the present study. The alga was grown routinely in Zarrouk's medium (1966) in a culture room maintained at 25±1°C temperature and illuminated with 3000 lux light intensity for 16 h light and 8 h dark cycle. Culture flasks were non-aerated and shaken manually several times a day. Microscopic observations of the algal samples were performed under light microscope (x 600) to identify the algal species and one Haemocytometer field was considered for calculating the tentative relative frequency of the algal species.

Measurement of growth and pigments of the alga - Increase in growth of alga was measured by optical density estimation at 660nm with junior spectrophotometer (E.C. Ltd. India). For dry weight estimation aliquotes of algal suspension were filtered on fine nylon net, washed with distilled water and dried overnight at 80°C before taking the weight.

For pigment analysis algal pellet was extracted in 90% methanol or 95% acetone in dark and monitered spectrophotometrically. Quantity of chlorophyll and carotenoid were estimated according to Machinney (1941). Phycocyanin pigments were extracted in 20mM phosphate buffer pH 7.0 by repeated freezing and thawing and quantity was determined by the method of Myers & Kratz (1955). Total protein of the alga was determined according to procedure of Lowry *et al.* (1951). Total carbohydrate and lipids were estimated according to procedure describes earlier (Saxena *et al.*, 1983).

Outdoor cultivation of *Spirulina* - Lucknow city sewage collected during morning hours was filtered through cotton pads to remove coarse suspended particles and used in laboratory as well as in out door cultivation. Erlenmeyer conical flasks were used for growing the alga with sewage under laboratory conditions. Out door cultivation of alga was carried in concrete constructed $2m^2$ basins operated at a depth of 15cm and stirred manually at regular intervals. During the experimental period the maximum day temperature was 36±1°C while minimum night temperature was 18±1°C and the maximum peak intensity during the day was 1600-1800 μmol m^2s^{-1}.

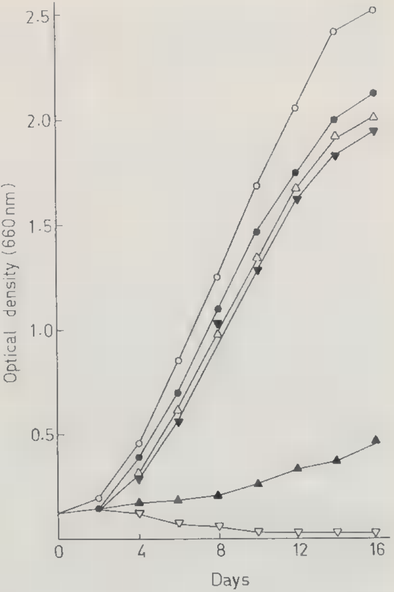

Fig. 1: Growth pattern of *Spirulina* in different medium under cultural conditions, Zarrouk basal medium (o-o); Sewage + all ingredients of Zarrouk medium (•-•); Sewage + N:P:K + all ingredients of Zarrouk medium except $NaNO_3$ (△-△); Sewage + N:P:K + HCO_3 (▼-▼); Sewage + NO_3 or HCO_3 (▲-▲); raw sewage only (▽-▽).

The commercial fertilizer N:P:K having combination of 20% nitrogen, 54% phosphate and 26% potassium was obtained from Indian Farm and Fertilizer company and used through out the experiment. To start the out door cultivation a thick slurry of the algal stock was transferred to sewage containing bicarbonate and nitrate or N:P:K. Apparently there was no significant loss of algal viability due to osmotic or pH shock on transferring the alga to sewage medium. These aspects of adaptation of *Spirulina* in sewage has been standardised in our earlier studies (Saxena *et al.*, 1981, 1983).

RESULTS AND DISCUSSION

Growth pattern of *Spirulina platensis* under controlled conditions in Zarrouk's basal medium showed a typical sigmoidal curve with an average final yield of 2-2.5 g/l dry weight. Direct transfer of alga from basal medium to raw and settled sewage in laboratory resulted into chlorosis and finally the lysis of the alga. To determine whether the lysis was due to the toxicity of sewage, or low pH (7.0-7.5) or deficiency of assimilable inorganic nutrients in sewage for algal growth. Therefore, the effect of fortifying the cultures with different combinations of inorganic nutrients were tested to know the algal growth-limiting nutrient in sewage (Fig. 1). In parallel set the raw sewage was supplemented with all the ingredients of the Zarrouk medium showed maximum growth comparable to the control (Zarrouk medium). This indicate that sewage does not contain any inhibitory effects on the growth of *Spirulina* and lysis of the alga in raw sewage is not due the toxicity of sewage. Addition of the ingredients of Zarrouk's medium to the sewage increased the initial pH (7-7.5) of sewage to 8-8.5. With increase in growth over the incubation period, pH of the sewage medium was increased to 10.5 after 2-3 days of growth. This is a normal phenomenon with photosynthetic organisms and is due to utilization of HCO_3 ions from the medium. These results are analogous to earlier report of Kosaric *et al.*, (1979) and suggest that *Spirulina* prefers higher pH in the growth medium. In order to minimize the fortification of chemicals in sewage, various constituents of Zarrouk medium were eliminated from the fortified sewage suspension one by one and the growth pattern and yield was determined. Results demonstrated that $NaNO_3$ as nitrogen and $NaHCO_3$ as carbon source were the essential compounds required in quantity for optimum growth of *Spirulina*. Replacement of the rest of the ingredients contributed only slightly to the final yield of alga. From these results it may be presumed that sewage contains most of the micro-nutrients and elements in quantities sufficient to support the growth except for macro-nutrients like No_3 and carbonate which are essential to maintain optimum pH and nitrogen level in the medium for growth of *Spirulina.*

In the attempt to determine the minimum quantities of nitrogen and carbon fortification are required for optimum growth and yield, sewage was supplemented in various combinations of $NaNO_3$ and $NaHCO_3$. Results summarized in Table I showed that with increasing concentrations of nitrate and bicarbonate growth yield was increased. A combination of 0.2 % nitrate and 1.5% bicarbonate showed maximum yield (2.2 g/l dry weight). However, in order to economize the supplementation a combination of 0.1% Na No_3 and 1% $NaHCO_3$ was selected. Results obtained with various combinations of bicarbonate and with N:P:K instead of $NaNO_3$ showed that the combination of 0.05% N:P:K and 1.5% $NaHCO_3$ resulted in yields of *Spirulina* comparable to nitrate suplemented cultures. The growth pattern and curve of *Spirulina* in sewage with bicarbonate and N:P:K was almost similar to NO_3 supplemented cultures. Concentration of N:P:K higher than above was found toxic for the growth of alga whereas concentration below 0.05% showed poor growth of the alga (Table I). These results also suggest that *Spirulina* is either not able to use the combined organic nitrogen available in the sewage or may be due to its high photosynthetic rate and strict photoautotrophic mode of growth the anaerobic bacteria are unable to degrade the combined nitrogenous substances into assimilable form. Whereas for cultivation of green algae in sewage such a high level of nitrogen fortification is not required (Shelef *et al.*, 1980).

Table I - Growth yield of *Spirulina platensis* (g/l dry weight) in sewage supplemented with different concentrations of $NaHCO_3$ and $NaNO_3$ or N:P:K

	NaHCO3 (%)				
	0.25	0.5	1	1.5	2.0
Na NO_3 (%)					
0.01	-	-	0.52	0.65	0.68
0.05	-	0.40	0.67	0.89	0.98
0.1	-	0.52	1.96	2.07	2.10
0.2	0.55	0.60	2.07	2.20	2.23
0.25	0.60	0.64	2.10	2.20	2.23
N:P:K (%)					
0.01	-	-	0.30	0.44	0.50
0.03	-	0.45	1.0	1.22	1.30
0.05	0.50	0.60	1.8	2.0	2.0
0.07	0.55	0.62	1.6	2.0	2.0
0.1	nt.	nt.	nt.	nt.	nt.

nt = no detectable growth was found.

The strains of *Spirulina* adapted in N:P:K supplemented medium under laboratory conditions were exposed to field conditions in sewage supplemented with 1% $NaHCO_3$ and 0.05% N:P:K. The outdoor cultivation of *Spirulina* is limited to several major factors like minerals, carbon-dioxide, pH, temperature, light and competing green algal species. Therefore, in attempt to adapt *Spirulina* in outdoor culture, a periodic observations of algal population, change in pH and major nutrients in sewage were monitored and results obtained are summarised in Table II. In the beginning several wild algae such as *Euglena, Phacus, Selenastrum, Ankistrodesmus, Scenedesmus, Chlorella, Phormidium, Myxosarcina, Chroococcus,* unidentified uni-cellular blue-green algae and diatoms were the dominant species. However, with increase of pH from 8.2 to 9.5 the wild algal species started disappearing while the population of *Spirulina* started increasing. After incubation of 7 to 8 days, pH of the medium increased between 9.5 to 10 and most of the wild species of green and blue-green algae except *Chlorella, Ankistrodesmus* and *Scenedesmus* were significantly reduced and *Spirulina* was the dominant species. After 6 to 8 successive subculturing *Spirulina* was adapted and grow luxuriantly as the most dominant species under field conditions.

For mass cultivation of *Spirulina* under field conditions a recharging schedule of two major nutrients was standardized to reduce the production cost of algal biomass. Results obtained are summarised in Fig. 2. From an open pond system harvesting of 10-20% of the algal suspension was done at intervals of 4 days, and the filterate was recycled back to the same culture to conserve the fortified chemicals. The concentration of sewage was maintained daily by the addition of fresh sewage to the ponds. Simultaneously the level of nitrate and bicarbonate was estimated in the filterate which showed that after 30-40 days the amount of N:P:K had decreased while the level of Na HCO_3 remained unchanged in the medium. This resulted in significant de-

Table II - Succession dynamics of algal species during acclimatisation of *Spirulina* under natural conditions in sewage fortified with 0.05% N:P:K and 1% $NaHCO_3$. D: dominants; V: very high frequency; H: high frequency; F: frequent; M: moderate frequency; L: low frequency; R: rare; N: not traceable.

	Days										
Algal species	0	2	4	6	8	10	12	14	16	18	20
Ankistrodesmus	L	F	M	M	F	F	F	F	F	L	L
Characium	L	L	F	F	L	R	R	R	N	N	N
Chlamydomonas	R	F	L	R	R	R	R	R	N	N	N
Chlorella	F	M	M	H	M	F	F	F	F	F	F
Chlorococcum	F	F	F	F	L	L	R	R	R	R	R
Chlorococcus	F	F	F	L	L	L	L	R	R	N	N
Diatoms	F	F	M	M	F	F	F	F	F	F	F
Euglena	M	M	M	F	F	L	L	L	L	L	L
Myxosarcina	F	F	F	F	F	F	F	F	F	F	F
Oocystis	R	L	F	F	R	R	R	R	N	N	N
Oscillatoria	L	F	F	F	L	L	R	R	N	N	N
Pediastrum	L	F	F	F	L	L	L	R	R	R	R
Phacus	F	F	F	F	L	R	R	R	R	R	R
Phormidium	F	F	F	F	F	L	L	R	R	R	R
Scenedesmus	F	M	M	M	F	F	F	F	F	F	F
Selenastrum	L	L	F	F	F	F	L	L	R	R	R
Spirulina	F	F	M	H	V	D	D	D	D	D	D
Unicellular blue-green algae	F	M	H	M	F	F	F	F	F	F	F
Trachelomonas	L	M	F	R	R	R	R	R	R	R	R
Zooplancton	F	M	M	M	M	F	F	F	F	F	F
pH of the sewage (extra cellular)	8.2	8.5	9.0	9.5	10	10	10.5	10.5	11	11	11

crease in the yield of the alga. Supplementation of N:P:K at this stage showed revival of growth and yield of *Spirulina platensis*. This suggested that under field conditions nitrate nutrient becomes a limiting source after 8 to 10 harvests and needs recharging whereas bicarbonate seems essential in maintaining the high pH, salinity and ionic equilibrium necessary for the optimum growth and sustained yield of alga. However, it is possible that under outdoor condition *Spirulina* may be simultaneously utilizing atmospheric CO_2 for growth, therefore, the level of HCO_3 is unaffected over the period of incubation (Richmond, 1985). With this recharging schedule a consistent yield of 1 to 1.2 g/l dry weight (6-6.5 g dry/m^2/day) can be obtained for total of 60-80 days in semi-continuous open pond system. Chemical analysis of the algal biomass obtained from sewage medium supplemented with either $NaNO_3$ or N:P:K is shown in Table III. Data showed that the level of protein, pigment and other macromolecules in N:P:K grown algal biomass was comparable to NO_3 supplemented cultures. From these studies it may be suggested that commercial fertilizer like N:P:K can replace expensive $NaNO_3$ and with a regular recharging schedule of N:P:K a consistent yield of alga can be obtained from semi-continuous open pond system. This strategy is considerably more economical that the use of expensive nitrogen sources.

Table III - Chemical composition (% dry wt.) of *Spirulina platensis* grown in sewage under identical conditions with different nitrogen sources.

	Sewage + bicarbonate + nitrate	Sewage + bicarbonate + N:P:K
Pigment composition		
Chlorophyll ■ -	0.76 - 0.94%	0.68 - 0.85%
Phycocyanin -	0.80 - 1.01%	0.72 - 0.90%
Carotenoids -	0.22 - 0.34%	0.19 - 0.35%
Crude protein -	50 - 55%	45 - 50%
Total carbohydrate -	18 - 20%	16 - 18%
Total lipids -	6.5 - 9.0%	5.9 - 7.3%

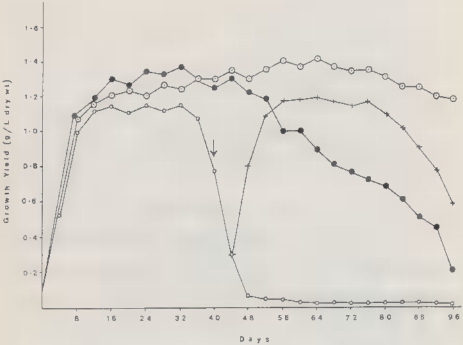

Fig. 2: Yield (g/l dry weight) of *Spirulina* under field conditions during periodic harvesting and recharging with ingredients. Cultures were initially supplemented with 0.05% N:P:K and 1% $NaHCO_3$ and harvesting of 1/10th of the total volume was performed at 4 days intervals and filterate recycled back (o-o); same as above followed by recharging with 0.05% N:P:K after 40 days as shown with arrow (+-+); same as above followed by successive recharging with 0.025% N:P:K after each harvest (•-•); no recycling of filterate and supplementation of fresh sewage, N:P:K and bicarbonate after each harvest (⊙-⊙).

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REFERENCES

BECKER E.W. & VENKETARAMAN L.V., 1984 - Production and utilization of the blue-green alga *Spirulina* in India. *Biomass.* 4: 105-125.

BURLEW J.S. (Ed.), 1953 - *Algal culture from laboratory to pilot plant.* Carnegie Institute of Washington Publication 600, Washington D.C.

CLEMENT G., 1975 - Production et constituants caractéristiques des algues *Spirulina platensis* et *maxima. Ann. Nutr. Alim.* 29: 477-488.

CLEMENT G., 1978 - Une nouvelle ressource: L'Algue *Spirulina. Soc. Phycol. France, Bull.* No 23: 57-61.

CHUNG Po., POND W.G., KINGSBURY J.M., WALKER Jr. E.F. & KROOK L., 1978 - Production and nutritive value of *Arthrospira platensis*, a spiral blue-green alga grown on swine wastes. *J. Anim. Sci.* 47: 319-330.

DURAND-CHASTEL H., 1980 - Production and use of *Spirulina* in Mexico. *In* SHELEF G. & SOEDER C.J. (Eds.), *Algae Biomass.* Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 51-64.

FAUCHER O., COUPAL B. & LEDUY A., 1979 - Utilization of seawater-urea as a culture medium for *Spirulina maxima. Canad. J. Microbiol.* 25: 752-759.

GOLDMAN J.C., 1979 - Outdoor algal mass cultures - I. Applications. *Water Res.* 13: 1-19.

KOSARIC N., NGUYEN H.T. & BERGOUGNOU M.A., 1979 - Growth of *Spirulina maxima* on cow-manure wastes. *Biotechnol. Bioengng.* 21: 2169-2173.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J., 1951 - Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

MACKINNEY G., 1941 - Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140: 315-322.

Mc GARRY M.G., 1971 - Unicellular protein production using domestic wastewater. *J. Agric. Sci.* 4: 213-223.

MYERS J. & KRATZ W.A., 1955 - Relation between pigment content and photosynthetic characteristics in a blue-green alga. *J. Gen. Physiol.* 39: 11-22.

ORON G., SHELEF G. & LEVI A., 1974 - Growth of *Spirulina maxima* algae in effluents from secondary waste-water treatment plants. *Biotechnol. Bioengn.* 16: 881-896.

OSWALD W.J., 1980 - Algal production - problems, achievements and potential. *In* SHELEF G. & SOEDER C.J. (Eds.), *Algae Biomass,* Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 1-8.

RICHMOND A.E., 1985 - *Handbook of microalgal mass culture.* CRC Press, Florida, USA.

SAXENA P.N., AHMAD M.R., SHYAM R. & AMLA D.V., 1981 - *Biotechnology of Spirulina cultivation in sewage.* Extension literature No 4, EBIS National Botanical Research Institute, Lucknow, India.

SAXENA P.N., AHMAD M.R., SHYAM R. & AMLA D.V., 1983 - Cultivation of *Spirulina* in sewage for poultry feed. *Experientia* 39: 1077-1083.

SHELEF G., AZOV Y., MORAINE R. & ORON G., 1980 - Algal mass production as an integral part of a wastewater treatment and reclamation system. *In* SHELEF G. & SOEDER C.J. (Eds.), *Algae Biomass,* Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 163-190.

SHELEF G. & SOEDER C.J., (Eds) 1980 - *Algae Biomass-production and use.* Elsevier/North-Holland Biomedical Press, Amsterdam.

SOEDER C.J., 1978 - Economic consideration concerning the autotrophic production of micro-algae at the technical scale. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* 11: 259-273.

SOONG P., 1980 - Production and development of *Chlorella* and *Spirulina* in Taiwan. *In* SHELEF G. & SOEDER C.J. (Eds.), *Algae Biomass.* Elsevier/North-Holland Biomedical Press Amsterdam., pp. 97-113.

TEL-OR E., BOUSSIBA S. & RICHMOND A.E., 1980 - Products and chemicals from *Spirulina platensis. In:* SHELEF G. & SOEDER C.J. (Eds.), *Algae Biomass,* Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 611-618.

ZARROUK C., 1966 - *Contribution à l'étude d'une cyanophyceae. Influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima (Setch. et Gardner) Geitler.* Ph. D. thesis. University of Paris, France.